

TRDL-1-GAMMA, A NOVEL TUMOR NECROSIS-LIKE LIGAND

1. RELATED APPLICATIONS

This application is related to and claims the benefit of United States Provisional  
5 Application Serial No. 60/157,913 of David A. Jones and Elizabeth Manos, filed October  
6, 1999 and entitled "TRDL-1, a Novel Tumor Necrosis-Like Ligand," which is  
incorporated herein by this reference.

2. FIELD OF THE INVENTION

10 The present invention relates to the isolation and characterization of a novel tumor  
necrosis-like ligand. More specifically, the invention relates to the isolation and  
characterization of Tumor Necrosis Factor Related Death Ligand-1 $\gamma$ , hereinafter TRDL-  
1 $\gamma$ .

15 3. TECHNICAL BACKGROUND

Tumor necrosis factor, hereinafter TNF, is the prototypic member of a family of  
cytokines with important roles in immune regulation, inflammation and cancer. Beutler,  
B. (1995), *J Investig Med.* 43: 227-235; Bazzoni, F. & Beutler, B., (1996) *N Engl J Med.*  
334: 1717-1725. The TNF family contains fourteen members in addition to TNF $\alpha$   
20 including lymphotoxin  $\alpha$  (Gray, P. W. et al. (1984), *Nature* 312: 721-724.), lymphotoxin  
 $\beta$  (Browning, J. L. et al. (1993), *Cell* 72: 847-856.), CD40L (Armitage, R. J. et al. (1992),  
*Nature* 357: 80-129.), CD30L (Smith, C. A. et al. (1993), *Cell* 73: 1349-1360.), CD27L  
(Goodwin, R. G. et al. (1993), *Cell*. 73: 447-450.), OX40L (Baum, P. R. et al. (1994),  
*Circ Shock*. 44: 30-34.), 4-1BBL (Goodwin, R. G. et al. (1993) *Eur J Immunol.* 23: 2631-  
25 2641.), FasL (Suda, T. et al. (1993), *Cell*. 75: 1169-1178.) TRAIL/APO-2L (Wiley, S. R.  
et al. (1995), *Immunity*. 3: 673-682; Pitti, R. M. (1996) *J Biol Chem.* 271: 12687-12690.),  
TL-1 (Tan, K. B. et al. (1997), *Gene*. 204: 35-46.), TRANCE/RANKL (Wong, B. R. et al.  
(1997), *J Biol Chem.* 272: 25190-25194; Anderson, D. M., et al. (1997), *Nature*. 390:  
175-9.), LIGHT (Mauri, D. N. et al. (1998), *Immunity*. 8: 21-30.), TWEAK  
30 (Chicheportiche, Y. et al. (1997), *J Biol Chem.* 272: 32401-32410.) and APRIL (Hahne,  
M., et al. (1996), *J Exp Med.* 188: 1185-1190.). These ligands are type II membrane  
associated proteins (excepting lymphotoxin  $\alpha$ ) that share primary structure similarities  
that are confined to their C-terminal regions. Ware, C. F. et al. (1996), *J Cell Biochem.*

60: 47-55. This ligand group interacts with a family of target transmembrane receptors that are defined by cysteine-rich extracellular domains. Bazzoni, F. & Beutler, B., (1996) *N Engl J Med.* 334: 1717-1725. In addition, several members of this receptor family including TNFR1, Fas, DR3, DR4 and DR5 contain a region of homology termed a  
5 "death domain," which couples receptor activation to the apoptotic cellular machinery. Ashkenazi, A. & Dixit, V. M. (1998), *Science.* 281: 1305-1308; Ashkenazi, A. & Dixit, V. M. (1999), *Curr Opin Cell Biol.* 11: 255-260; Chinnaiyan, A. M., et al. (1996), *Science.* 274: 990-992; Marsters, S. A. et al. (1996), *Curr Biol.* 6: 1669-1676; Sheridan, J. P. et al. (1997), *Science.* 277: 818-821; Pan, G. et al. (1997), *Science.* 277: 815-818; Pan,  
10 G. et al. (1997), *Science.* 276: 111-113. The number of receptors contained within this family currently exceeds the number of putative ligands and suggests the existence of previously uncharacterized ligands. Identification of the cognate ligand for each receptor is necessary before a complete understanding of their roles in disease can be fully appreciated.

15 The importance of this cytokine family to immune system function is highlighted by phenotypic alterations associated with gene knockout experiments and endogenous mutations in mice. Mutations disrupting FasL or its counterpart receptor cause lymphadenopathy and autoimmune disorders. Watanabe-Fukunaga, R. et al. (1992), *Nature.* 356: 314-317; Adachi, M. et al. (1993), *Proc Natl Acad Sci U S A.* 90: 1756-  
20 1760; Takahashi, T. et al. (1994), *Cell.* 76: 969-976; Rieux-Laucat, F. et al. (1995), *Science.* 268: 1347-1349; Fisher, G. H. et al. (1995), *Cell.* 81: 935-946. In another example, CD40 mutations lead to hyper-immunoglobulin M phenotypes suggesting an essential role for the CD40 pathway in B cell affinity maturation and isotype switching. Allen, R. C. et al. (1993), *Science.* 259: 990-993. Inactivation of the TNF receptor type I  
25 pathway through knockout strategies generates mice that are highly sensitive to certain microbial infections, suggesting an important host defense role for TNF. Pfeffer, K. et al. (1993), *Cell.* 73: 457-467; Rothe, J. et al. (1993), *Nature.* 364: 798-802. Lastly, inactivation of the murine lymphotoxin gene causes loss of peripheral lymph nodes. De Togni, P. et al. (1994), *Science.* 264: 703-707.

30 Diverse roles for these cytokines are likely to extend beyond strict regulation of immune system function and into areas such as tumor development. A number of studies have demonstrated high levels of Fas ligand expression in human tumors. Primary

astrocytic brain tumors, colonic adenocarcinomas and metastases of human colonic adenocarcinomas all show increased levels of Fas ligand expression as compared to control tissues. Beg, A. A. & Baltimore, D. (1996), *Science*. **274**: 782-784; Shiraki, K. et al. (1997), *Proc Natl Acad Sci USA*. **94**: 6420-6425; Niehans, G. A. et al. (1997), *Cancer Res.* **57**: 1007-1012. Over expression of these ligands could contribute to tumor development from two perspectives. First, overexpression of death inducing ligands, such as FasL, may provide a defense that protects tumor cells by killing intervening host immune cells. Walker, P. R. et al. (1997), *J. Immunol.* **158**: 4521-4524; Whiteside, T. L. & Rabinowich, H. (1998), *Cancer Immunother.* **46**: 175-184; Bennett, M. W. et al. (1999), *Gut*. **44**: 156-162; Walker, P. R. et al. (1998), *Curr Opin Immunol.* **10**: 564-572. Secondly, expression of TNF family ligands in tumors could create a chronic inflammatory condition that renders tumor cells resistant to immune destruction and confounds chemotherapeutic approaches that rely on tumor cell apoptosis. Evidence for this resistance can be seen in the observation that TNF fails to kill many types of cancer cells. Recent evidence suggests that TNF undermines its own killing powers by activating NF-kB, a key molecule that can block the apoptosis pathway. Van Antwerp, D. J. et al. (1996), *Science*. **274**: 787-789; Wang, C. Y. et al. (1996), *Science*. **274**: 784-787. Disruption of this protective mechanism may, therefore, sensitize cells to chemotherapeutic intervention.

Current interventional strategies targeting TNF pathways are challenged by a lack of understanding of how these pathways are regulated and dysregulated in disease. Furthermore, an incomplete roster of ligands and receptors adds to the complexity of selecting rational points for intervention.

From the foregoing, it will be appreciated that it would be an advancement in the art to identify and characterize nucleic acid sequences that code for ligands within the TNF family of cytokines. It would be a further advancement if the nucleic acid sequences could provide additional understanding of how TNF pathways are regulated and dysregulated in disease.

Such nucleic acid sequences and methods are disclosed and claimed herein.

#### 4. BRIEF SUMMARY OF THE INVENTION

The present invention relates to a novel human alternatively spliced Tumor Necrosis-Like Ligand (TRDL). Provided herein are nucleic acid molecules that encode such TRDL molecules. The nucleic acid molecules of the present invention may also  
5 comprise the nucleotide sequence for human TRDL-1 $\gamma$  (SEQ ID NO: 1). In certain other embodiments, the present invention provides nucleic acid molecules that code for the amino acid sequence of human TRDL-1 $\gamma$  (SEQ ID NO: 2). The invention also provides nucleic acid molecules complimentary to the nucleic acid molecule of SEQ ID NO: 1.

The present invention also provides recombinant vectors comprising nucleic acid  
10 molecules that code for TRDL-1 $\gamma$ . These recombinant vectors may be plasmids. In other embodiments, these recombinant vectors are prokaryotic or eukaryotic expression vectors. The nucleic acid coding for TRDL-1 $\gamma$  may also be operably linked to a heterologous promoter.

The present invention further provides host cells comprising a nucleic acid that  
15 codes for TRDL-1 $\gamma$ .

These and other advantages of the present invention will become apparent upon reading the following detailed description and appended claims.

#### 5. SUMMARY OF THE DRAWINGS

20 A more particular description of the invention briefly described above will be rendered by reference to the appended drawings and graphs. These drawings and graphs only provide information concerning typical embodiments of the invention and are not therefore to be considered limiting of its scope.

Figure 1 shows the Primary Amino Sequences and Alignment of APRIL/TRDL-  
25 1 $\alpha$  (SEQ ID NO: 4); TRDL-1 $\beta$  (SEQ ID NO: 5); and TRDL-1 $\gamma$  (SEQ ID NO 2). The predicted primary amino acid sequences of TRDL-1 $\alpha$ , TRDL-1 $\beta$  and TRDL-1 $\gamma$  were aligned by the Clustal method. Areas where TRDL-1 $\alpha$  differs from TRDL-1 $\beta$  and TRDL-1 $\gamma$  are boxed.

Figure 2A illustrates tissue distribution of APRIL/TRDL-1. Expression of  
30 TRDL-1 in normal human tissues was evaluated by northern analysis of mRNA derived from eight human tissues (lane 1, spleen; lane 2, thymus; lane 3, prostate; lane 4, testis;

lane 5, ovary; lane 6, small intestine; lane 7, colon; and lane 8, peripheral blood leukocytes).

Figure 2B is a bar graph illustrating tissue distribution of APRIL/TRDL-1. Dot blot analysis was performed on mRNA isolated from 50 human tissues. Signal intensities were quantified using a phosphorimager, normalized for mRNA loading and are displayed as relative fluorescence.

Figure 3A is a bar graph illustrating expression of APRIL/TRDL in cancer cell lines and human tumor specimens. Expression of TRDL-1 was examined in eight human cancer cell lines (*bar 1, HL-60; bar 2, HeLa S3; bar 3, K-562; bar 4, MOLT-4; bar 5, Raji; bar 6, SW480; bar 7, A549; bar 8, G361*) by northern analysis. Hybridization signals were quantified using a phosphorimager and are displayed in the plot as relative phosphorescence.

Figure 3B is a bar graph illustrating expression of APRIL/TRDL in cancer cell lines and human tumor specimens. A multiple human tumor blot containing 48 tumor and corresponding normal tissues was hybridized with a TRDL-1 specific cDNA probe. Hybridization signals were quantified using phosphorimager, and a portion are displayed as relative phosphorescence.

Figure 3C is a bar graph illustrating expression of APRIL/TRDL in cancer cell lines and human tumor specimens. A multiple human tumor blot containing 48 tumor and corresponding normal tissues was hybridized with a TRDL-1 specific cDNA probe. Hybridization signals were quantified using phosphorimager, and a portion are displayed as relative phosphorescence.

Figure 4 illustrates expression, subcellular localization and purification of TRDL-1. COS-1 cells were transfected with pFLAG/TRDL-1 $\alpha$  (lanes I and 2) and cells harvested 48 hours later. Membrane (M) and (C) cytosolic fractions were examined for FLAG/TRDL-1 expression using antibodies specific for FLAG epitope (right) or TRDL-1 (left). (B) Cos-I cells ( $1 \times 10^8$ ) were transfected with pFLAG/TRDL-1 and incubated for 96 h. Following incubation cell membranes were prepared by hypotonic lysis. FLAG/TRDL-1 was solubilized from the membrane in a buffer containing 10% glycerol and 1% Triton-X100. Solubilized TRDL-1 was adsorbed onto an anti-FLAG M2 affinity column and eluted using 0.1 M free FLAG peptide. Fractions containing TRDL-1 were identified by immunoblotting

and combined. A portion was run on a 10% gel and silver stained to visual TRDL-1 enrichment.

Figure 5A illustrates in vitro binding of purified TRDL-1 to receptors from the TNF superfamily. Control anti-FLAG M2 affinity beads or beads harboring approximately 25 ng of TRDL-1 were combined with 250 ng of each candidate receptor/Fc fusion protein including: FAS/Fc (lane 1), TNFR1/Fc (lane 2), HVEM/Fc (lane 3), TR1/Fc (lane 4), TR2/Fc (lane 5) or TR3/Fc (lane 6). Binding reactions were incubated at 4°C for 2 h and unbound proteins removed by four consecutive washes with binding buffer. Remaining proteins were eluted by boiling in SDS-PAGE sample buffer and loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes and the blots probed using an antibody specific for the Fc portion of the receptor fusions.

Figure 5B is a bar graph illustrating the specific binding of TRDL-1 relative to control beads. Signals in and were quantified by scanning densitometry and TRDL-1 specific binding presented relative to binding in control beads.

Figure 6A is a photograph illustrating TRDL-1 induction of Jurkat cell apoptosis. Cultures of Jurkat cells ( $5 \times 10^4$ ) were incubated with vehicle, 50 ng/ml FasL, 50 ng/ml TNF, or 1.0 µg/ml TRDL-1α. Following incubation, cell death was assessed by visual inspection.

Figure 6B is a bar graph illustrating TRDL-1 induction of Jurkat cell apoptosis obtained by fluorescence monitoring of the uptake of YOPRO-1 dye. Dye uptake was quantified using a fluorescence plate reader and data presented as arbitrary fluorescence units.

Figure 7A is a bar graph illustrating saturable killing of Jurkat cells by TRDL-1. Cell counts are presented as mean  $\pm$  S.D.

Figure 7B is a concentration curve illustrating saturable killing of Jurkat cells by TRDL-1. The concentration curve for TRDL-1α killing was performed by incubating triplicate cultures of Jurkat cells ( $5 \times 10^4$ ) with 0-1000 ng/ml FLAG/TRDL-1α for 48 hours. Following incubation, cell number was determined by counting with a Coulter counter. Cell counts are presented as % control to show saturation.

## 6. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to cDNA and genomic clones for a novel Tumor Necrosis-Like Ligand (TRDL). More particularly, the present inventions relates to the

isolation and characterization of TRDL-1 $\gamma$  (SEQ ID NO: 1). TRDL-1 $\gamma$  expression in HeLa and SW4810 cells as well as in gastrointestinal tumors, including rectum, duodenum, colon, stomach, and esophagus suggests that this TRDL-1 may have a role in tumor development.

5 A nucleotide sequence complimentary to the nucleotide sequence of SEQ ID: 1 is also provided. Nucleic acid molecules that code for the amino acid sequence of the human TRDL-1 $\gamma$  protein (SEQ ID NO: 2) are also within the scope of the invention.

The nucleic acid molecules that code for TRDL-1 $\gamma$  can be contained within recombinant vectors. Recombinant vectors may be, e.g., plasmids, recombinant phages or viruses, transposons, cosmids, or artificial chromosomes. Such vectors may further include  
10 elements that control the replication and expression of the TRDL-1 $\gamma$  nucleic acid sequences. Such vectors may also include sequences (such as antibiotic resistance genes) that allow for the screening or selection of cells containing the vector. In certain embodiments of the invention, recombinant vectors of the present invention are plasmids. These recombinant vectors can be prokaryotic expression vectors or eukaryotic expression vectors. The nucleic  
15 acid coding for TRDL-1 $\gamma$  can be operably linked to a heterologous promoter.

The present invention further provides host cells comprising a nucleic acid that codes for TRDL-1 $\gamma$ . Such host cells may be prepared by transfecting an appropriate nucleic acid into a cell using transfection techniques that are known in the art. These techniques include, e.g., calcium phosphate co-precipitation, microinjection, electroporation, liposome-mediated  
20 gene transfer, and high velocity microprojectiles.

A method for the identification of a cell line that undergoes apoptosis upon interaction with TRDL-1 $\gamma$  also is provided. First, a culture of a mammalian cell line is divided into a test culture and a control culture. The test culture is contacted with a TRDL-1 $\gamma$  polypeptide and the control culture is not contacted with a TRDL-1 $\gamma$  polypeptide. Next, a  
25 determination is made of the quantity of cells of the test culture and the control culture that have undergone apoptosis. These quantities are compared. The determination that more cells have undergone apoptosis in the test culture indicates that the mammalian cell line undergoes apoptosis upon interaction with TRDL-1 $\gamma$ .

The invention also provides a method for the identification of agents capable of  
30 inhibiting or enhancing TRDL-1 $\gamma$  mediated induction of apoptosis. First, a mammalian cell line that has been determined to undergo apoptosis is obtained. The cells are divided into a test culture and a control culture. The cells of the test culture are contacted with a TRDL-1 $\gamma$

polypeptide in the presence of a test agent. The cells of the control culture are contacted with a TRDL-1 $\gamma$  polypeptide in the absence of the test agent. Next, a determination is made of the quantity of cells of the test culture and the control culture that have undergone apoptosis. These quantities are compared. The determination that fewer cells have undergone apoptosis in the test culture than in the control culture indicates that the test agent inhibits TRDL-1 $\gamma$  mediated induction of apoptosis. The determination that a higher quantity of cells have undergone apoptosis in the test culture than in the test culture indicates that the agent enhances TRDL-1 $\gamma$  mediated induction of apoptosis

In order to better describe the details of the present invention, the following discussion is divided into six sections: (1) identification and cloning of TRDL-1 $\alpha$ , TRDL-1 $\beta$ , and TRDL-1 $\gamma$ ; (2) tissue distribution of TRDL-1; (3) chromosomal localization of TRDL-1; (4) subcellular localization and purification of TRDL-1 $\alpha$ ; (5) association with TNF family receptors *in vitro*; and (6) induction of Jurkat cell death by FLAG/TRDL-1 $\alpha$ .

#### 6.1 Identification and Cloning of TRDL-1 $\alpha$ , TRDL-1 $\beta$ , and TRDL-1 $\gamma$

The peptide sequence of human tumor necrosis factor  $\alpha$  and the BLAST (basic local sequence alignment tool) algorithm to query the dbEST database was used in order to identify novel members of the tumor necrosis factor family of cytokines. Altschul, S. F. et al. (1990), *J Mol Biol.* **215**: 403-10. An initial search identified seven EST sequences that showed statistically relevant ( $P < 1.0$ ) alignments with TNF  $\alpha$  and were derived from human tissue sources. Of these seven, two sequences, AA405973 and AA477087, displayed interesting homology with TNF $\alpha$  in regions where TNF $\alpha$  is also similar to FasL, TRAIL and other members of the TNF cytokine family (data not shown).

This conserved region aligns with a beta-pleated strand of TNF $\alpha$  (the D strand) that is central to ligand homotrimerization based on crystallographic studies. Eck, M. J. & Sprang, S. R. (1989), *J Biol Chem.* **264**: 17595-17605; Eck, M. J. et al. (1992), *J Biol Chem.* **267**: 2119-2122. To pursue this observation, the database was mined further and ten additional ESTs were identified. These additional ESTs had sequences that overlapped AA405973 and AA477087. This set of ESTs defined a cluster that continued to share sequence similarity with the TNF family. Each of the clones identified was part of the IMAGE consortium and was readily obtained from an IMAGE consortium distributor. Nucleotide sequence analysis of each clone confirmed an initial, computer-based alignments



and strengthened the assignment of this cDNA into the TNF family (Figure 1). Of the ten ESTs examined, clone 727332 contained 1260 bp and a single, continuous open reading frame that predicted a 250 amino acid protein with a molecular mass of 27,432 daltons. Primary alignment of the predicted open reading frame with TNF $\alpha$  extended the critical  
5 homology regions and the clone was renamed TRDL-1 (Tumor necrosis factor-Related Death Ligand-1) to identify it as new member of the TNF family. Secondary structure analyses predicted that TRDL-1 contains a short cytoplasmic domain (residues 1-27), a transmembrane helix (residues 28-50) and a 199 amino acid extracellular domain (residue 51-250).

10 Although the sequence of TRDL-1 encoded by clone 727332 was convincing enough to assign it to the TNF family, clone 727332 lacked a poly adenylation signal and poly A+ tail. This required additional cDNA cloning experiments to identify a full-length TRDL-1 cDNA. To isolate full-length TRDL-1 cDNAs, a human leukocyte cDNA library was screened using TRDL-1 as a probe. Primary screening of  $5 \times 10^5$  plaques on duplicate filters  
15 identified twelve clones that were carried through secondary and tertiary screens. A total of eleven clones survived secondary and tertiary screening efforts with the TRDL-1 probe and were plaque purified. Restriction analysis of the eleven clones grouped them into two types of cDNAs ( $\beta$  and  $\gamma$ ) that appeared to differ in organization from TRDL-1. Sequence determination of clones representing each of these variants confirmed their identity with  
20 TRDL-1 and revealed potential alternative splicing products (Figure 1). TRDL-1 $\beta$  was a 1684 bp clone that contained an open reading frame that predicted a protein of 234 amino acids and a molecular mass of 25,677 daltons. By comparison TRDL-1 $\gamma$  was composed of 1607 bp and predicted a 257 amino acid protein with a molecular mass of 27057 daltons. TRDL-1 $\beta$  and TRDL-1 $\gamma$  each contained a polyadenylation signal and a polyA+ tail. Figure  
25 1A shows the aligned, predicted amino acid sequences of TRDL-1 $\alpha$ , TRDL-1 $\beta$  and TRDL-1 $\gamma$ . TRDL-1 $\beta$  was identical to TRDL-1 $\alpha$  with the exception of a 48 bp deletion that removed 16 amino acids corresponding to residues 113 through 128 of TRDL-1 $\alpha$ . TRDL-1 $\gamma$  was also largely identical to TRDL-1 $\alpha$  but contained a 3' deletion of 181 bp that results in substitution of the four C-terminal residues of TRDL-1 $\alpha$  with a single leucine residue.

## 6.2 Tissue Distribution of TRDL-1

Next, the tissue distribution of TRDL-1 was examined by performing northern analyses on mRNA from various human tissues. Hybridization with TRDL-1 $\alpha$  identified mRNA species in most tissues. Highest levels of expression were seen in peripheral blood leukocytes, with intermediate levels of expression noted in pancreas, colon, small intestine, prostate and ovary (Figure 2A). There was little expression in skeletal muscle, thymus, or testis. While most tissues expressed a 1.8 kb mRNA, peripheral blood leukocytes and lung expressed a message of 1.6 kb. Dot blot analysis of 34 additional tissues showed low levels of TRDL-1 expression in most tissues (Figure 2B).

To assess a potential role for TRDL-1 in tumor development, its expression in cancer cell lines was examined (Figure 3A). Strong expression of TRDL-1 (1.8 kb species) was observed in mRNA from HeLa and SW4810 cells. Expression was undetectable in the other cancer cell lines examined and suggested the potential for cell type specific regulation of TRDL-1 in cancers. The cancer cell-based observations were extended, by surveying 48 human tumor biopsies compared to normal tissues for the expression of TRDL-1 (Figure 3B and 3C). A number of tumors showed higher levels of TRDL-1 expression as compared to adjacent normal tissues. Of note, were gastrointestinal tumors, including rectum, duodenum, colon, stomach and esophagus.

## 6.3 Chromosomal Localization of TRDL-1

To determine the chromosomal localization of TRDL-1, a BAC clone containing the TRDL-1 gene was isolated to serve as a probe for fluorescent *in situ* hybridization against PHA treated peripheral blood leukocytes. An initial experiment with the TRDL-1/BAC resulted in labeling of the short arm of a group E chromosome that was believed to be chromosome 17 on the basis of size, morphology and banding pattern. A second experiment was conducted with a probe specific for the centromeric region of chromosome 17 that cohybridized with TRDL-1. This experiment demonstrated the specific labeling of the chromosome 17 centromere in red and the short arm of chromosome 17 in green. Measurement of ten specifically labeled chromosomes 17 demonstrated that TRDL-1 is located at a position 77% of the distance from the centromere to the telomere of chromosome arm 17p in an area which corresponds to band 17p13.3. A total of 80 metaphase cells were analyzed with 72 exhibiting specific labeling (data not shown).

#### 6.4 Subcellular Localization and Purification of TRDL-1 $\alpha$

Secondary structure predictions for TRDL-1 suggested a single membrane-spanning region near the N-terminus. To verify membrane localization, next the subcellular  
5 localization of FLAG-tagged TRDL-1 $\alpha$  in COS-1 cells was examined. To accomplish this, FLAG/TRDL-1 $\alpha$ , was transiently expressed in COS-1 cells, and its distribution in cytosolic or membrane fractions examined by western analysis using antibodies specific for the FLAG epitope or TRDL-1. Figure 4A shows the majority of FLAG and TRDL-1 cross re-activities in the membrane fractions of cells transfected with TRDL-1.

10 Then FLAG/TRDL-1 $\alpha$  from  $1 \times 10^8$  COS-1 cells that had been transfected with pFLAG/TRDL-1 $\alpha$  was purified. Following a 96 hour incubation, cell membranes were prepared by hypotonic lysis and differential centrifugation. Portions of membrane preparations were then combined with a solubilization buffer containing 10% glycerol and 1% Triton-X-100. Greater than 90% of the TRDL-1 protein was solubilized under these  
15 conditions. Solubilized TRDL-1 was then passed over the anti-FLAG M2 affinity gel to facilitate purification. The FLAG-tagged TRDL-1 protein was completely removed by the affinity matrix and was successfully eluted with 0.1 M FLAG peptide. Figure 4B shows a silver stained gel of purified FLAG/TRDL-1 and cross reactivity of the purified protein with antibodies specific for the FLAG epitope and TRDL-1. The purification of FLAG/TRDL-1  
20 was estimated at greater than 80%.

#### 6.5 Association with TNF family Receptors *In Vitro*

Since TRDL-1 showed structural similarities to TNF family members, it was reasoned that TRDL-1 may bind to TNF family receptors. Therefore, the ability of TRDL-1 $\alpha$   
25 to interact with purified TNF family receptors *in vitro* was examined. This was performed by capturing FLAG/TRDL-1 $\alpha$  onto anti-FLAG affinity beads and combining these beads with purified TNFR1/Fc, FAS/Fc, HVEM/Fc, TR1/Fc, TR2/Fc, and TR3/Fc fusion proteins. Precipitation of each receptor was assessed by immunoblotting for the Fc portion of the receptor fusions. Figure 5A shows FLAG/TRDL-1 $\alpha$  mediated precipitation of known TNF  
30 family receptors. Although there was detectable binding to all receptors above background, densitometric scanning revealed strongest binding of FLAG/TRDL-1 $\alpha$  to Fas (10.1 fold above

background) and HVEM (11.2 fold above background) as compared to control beads and to the other receptors (Figure 5B).

#### 6.6 Induction of Jurkat Cell Death by FLAG/TRDL-1 $\alpha$

5 Following the observation that FLAG/TRDL-1 $\alpha$  could bind to TNF family receptors *in vitro*, it was assessed whether FLAG/TRDL-1 $\alpha$  stimulated apoptosis in Jurkat cells. Figure 6A shows the morphological consequences of exposing Jurkat cells to vehicle alone, 50 ng/ml FasL, 50 ng/ml TNF $\alpha$  or 1.0  $\mu$ g/ml TRDL-1 for 16 h. FasL and TRDL-1 each caused clumps of Jurkat cells, to disperse as compared to vehicle-treated cells or cells treated  
10 with TNF $\alpha$ . The dispersal of Jurkat cells parallels cell death markers like caspase activation and annexin staining and suggest activation of similar pathways by TRDL-1 and FasL. Further inspection of the FasL and TRDL-1-treated cells showed typical apoptotic markers including nuclear condensation, membrane blebbing and cell shrinkage. In contrast, vehicle- and TNF-treated cells showed few of these characteristics at 16 hours post-treatment. In  
15 these same cells, viability was assessed by YOPRO-1 dye uptake. Figure 6B shows stimulation of YOPRO-1 dye uptake in cells treated with Fas, TRDL-1 for 12 hours but not in cells treated with vehicle and TNF $\alpha$ . Finally, depletion of TRDL-1 by immunoprecipitation using the TRDL-1 specific antisera eliminated the death-inducing capability and confirmed TRDL-1 as the active protein in the preparation (data not shown).  
20 Finally, a concentration curve for TRDL-1 induced death was established by incubating Jurkat cells with 0-1000 ng/ml TRDL-1 for 48 hours. Following incubation, cell numbers were quantified by counting on a Coulter cell counter. Figure 7A illustrates the concentration-dependent decline in cell number in response to TRDL-1. Re-plotting of this data revealed that TRDL-1-induced death is saturable (Figure 7B). Maximal activity of  
25 TRDL-1 was seen at concentrations above 100 ng/ml with TRDL-1 showing an ED<sub>50</sub> of approximately 50 ng/ml.

#### Materials and Methods

30 Cell Culture Reagents: Jurkat and Cos-1 cells obtained from ATCC. (American Type Culture Collection) were grown in RPMI-1640 and DMEM respectively. All media was supplemented with 10% fetal bovine serum, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 50 units/ml penicillin G sodium and 50  $\mu$ g/ml streptomycin sulfate. Cells were grown at

37°C under 5% CO<sub>2</sub>. All media and supplements were obtained from Life Technologies, Inc. (Gibco/BRL).

All patents, publications, and commercial materials cited herein are hereby incorporated by reference.

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## 7. EXAMPLES

The following examples are given to illustrate various embodiments which have been made with the present invention. It is to be understood that the following examples are not comprehensive or exhaustive of the many types of embodiments which can be prepared in accordance with the present invention.

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### **Example 1 - TRDL-1 Cloning and Sequence Determination**

Clones corresponding to potential TNF $\alpha$  matches were obtained from GenomeSystems, Inc. IMAGE clone 727332 (TRDL-1 $\alpha$ ) was used to screen a human leukocyte cDNA library (Clontech Laboratories, Inc.). After an initial titering of the library, a total of 500,000 plaques were lifted in duplicate onto nylon filters. Phage DNA was denatured with the following wash protocol: 2X SSC for 1 min; 1.5 M NaCl, 0.5 N NaOH for 2 min, 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 2 min and 2X SSC for 2 min. Filters were air-dried and UV-cross-linked using a Stratalinker (Stratagene). Filters were prehybridized in 6X SSC, 1X Denhardt's solution, 100  $\mu$ g/ml denatured salmon sperm DNA and 0.5% SDS for 2.0 h at 65°C to block nonspecific DNA binding sites.

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The probe was labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP (Amersham) to a specific activity of greater than 1 X 10<sup>9</sup> cpm/ $\mu$ g of DNA using an RTS RadPrime DNA labeling System (Gibco/BRL). Unincorporated nucleotides were removed by passage over Chroma-Spin-30 (Clontech Laboratories, Inc.) size exclusion columns. Hybridization was performed in the same buffer as prehybridization for 12-14 h at 65°C with a probe concentration of 1 x 10<sup>6</sup> cpm/ml. Following hybridization, unbound probe was removed by washing filters twice for 20 min each in 1 X SSC, 0.1% SDS at room temperature followed by 2 washes for 20 min each in 0.1 X SSC, 0.1% SDS at 65°C. Filters were exposed to X-ray film for 12-14 h with intensifying screens at -70°C. Plaques that showed duplicate hybridization signals were selected into an appropriate phage buffer and titered. Each positive plaque was then subjected to two additional rounds of hybridization screening to completely isolate the

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positive plaques from other species and to eliminate false positives. DNA was isolated and insert size determined from plaques resulting from tertiary screens. Clones were sequenced by the Huntsman Cancer Institute Core DNA Sequencing Facility using ABI Prism BigDye Terminators and cycle sequencing with Taq FS DNA Polymerase. DNA Sequence was  
5 collected and analyzed on an ABI Prism 377 automated DNA sequencer (PE Applied Biosystems Division, Foster City, California).

### Example 2 - Northern and Dot Blot Analyses

Multiple human tissue mRNA blots I and II a human cancer cell line blot, and a  
10 human RNA dot blot were purchased from Clontech Laboratories, Inc. A multiple tumor mRNA blot was obtained from Biochain Institute, Inc. The membranes were prehybridized in ExpressHyb™ (Clontech Laboratories, Inc.) for 2.5 h at 65°C then hybridized with a random-primed <sup>32</sup>P-labeled TRDL-1 probe for 2.5 h at 65°C. Unbound probe was removed by washing twice at room temperature in 2x SSC/0.05% SDS and twice at 50°C in 0.1x  
15 SSC/0.1%SDS. To quantify hybridization signals, blots were exposed to a phosphorimager (Molecular Dynamics) screen for 6-24 h.

### Example 3 - Chromosomal Localization

A TRDL-1 BAC DNA was labeled with digoxigenin-dUTP by nick translation. The  
20 labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin, (PHA)-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2X SSC. Specific hybridization signals were detected by incubation with fluoresceinated antidigoxigenin antibodies followed by counterstaining with 4', 6-diamidine-2'-phenylindole  
25 dihydrochloride for one color experiments.

### Example 4 - Expression of FLAG/TRDL-1 in Cos-1 cells

Full-length TRDL-1α was cloned into pFLAG-CMV-2 (Kodak) for expression in  
Cos-1 cells. Transient transfections were performed using Lipofectamine reagent  
30 (Gibco/BRL) according to manufacturer's protocol. Briefly, 10 μg of DNA was combined with 50 μl of Lipofectamine in 5.0 ml of serum-free Optimem (Gibco/BRL). Following DNA/Lipofectamine complex formation, the transfection mixture was added to 80%

confluent Cos-1 cells in a 100 mm culture dish. After 4 h incubation under standard conditions, 5 ml of DMEM/10% fetal bovine serum was added and cells allowed to recover overnight. On the following day, the medium was removed and replaced with fresh, complete culture medium. TRDL-1 expression was monitored at approximately 48 hours post transfection by analyzing cellular lysates for FLAG epitope.

#### **Example 5 - Membrane Solubilization and Purification of FLAG/TRDL-1**

Cos-1 cells transiently transfected with full-length TRDL-1 were incubated for 1 h at 4 °C in hypotonic lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM PMSF, 10µg/ml leupeptin and 10µg/ml aprotinin). Cell lysates were centrifuged at 500 x g for 7 min to remove the nuclei. The supernatant was then spun at 3000 x g for 15 min to pellet the membranes. Membrane fractions were washed twice with cold PBS and solubilized in a buffer consisting of 1% Triton-X-100, 50 mM Tris pH 7.4, 150 mM NaCl and 10% glycerol overnight at 4°C. Particulate was removed by centrifugation at 12,000 x g for 10 min. Protein samples were loaded onto an anti-FLAG (M2) affinity resin (Kodak) at a flow rate of 0.2 ml/min. Columns were washed with 5 volume equivalents of PBS/0.1% Tween-20 and 5 volume equivalents of 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, and 10% glycerol. FLAG-TRDL-1 was eluted off the column by competition with 0.1 M FLAG peptide (Sigma). Fractions were assayed for the presence of TRDL-1 by immunoblotting. Fractions containing TRDL-1 were pooled and concentrated via a Centricon-10 (Amicon). Purity of the eluate was determined by SDS-polyacrylamide gel electrophoresis and silver staining (Bio-Rad).

#### **Example 6 - Generation of TRDL-1 Antibodies**

A synthetic 15-amino acid peptide corresponding to residues 121-135 of TRDL-1α was used to generate rabbit polyclonal antibodies. The peptide, CPINATSKDDSDVTE (Seq Id. No: 3), was conjugated to keyhole limpet hemocyanin (KLH) and rabbits were immunized by Quality Controlled Biochemicals, Inc. Test bleeds were titrated by ELISA and tested by Western blot against a whole cell lysate from Cos-1 cells expressing recombinant FLAG/TRDL-1. Sera showing good cross-reactivity were affinity purified to produce 5 mg of purified anti-TRDL-1 antibody.

### Example 7 - Immunoblot Analysis

Samples were assayed for protein concentration using Bio-Rad protein assay kit. Protein samples (5-10  $\mu$ g) were analyzed by SDS-PAGE on 10% tricine gels (Novex) and electro-transferred to PVDF (polyvinylidene difluoride) membranes (Gelman Sciences). Blots were incubated for 1 h at room temp in blocking buffer (5% powdered milk/PBS/0.1% Tween-20) then with a monoclonal antibody to the FLAG epitope (Kodak) diluted 1:10,000 in blocking buffer for 1 h at room temp or overnight at 4°C. Blots were washed 3x in PBS/0.1% Tween then probed with a secondary antibody conjugated to horseradish peroxidase. After washing 3x in PBS/0.1% Tween, FLAG protein was detected using a chemiluminescent substrate (NEN) according to manufacturer's instructions.

### Example 8 - Receptor Binding Assays

All steps were carried out at 4°C. Sepharose beads coated with M2 antibody (anti-FLAG, Kodak) were blocked in 1% BSA for 1 h. After blocking, lysates (see above) from TRDL-1 transfected Cos-1 cells and untransfected cells were incubated with the blocked beads for 1 h. The beads were washed to remove detergent and unbound protein and then incubated with 250 ng of recombinant purified receptors fused to Fc (R&D Systems) for 2 h at 4°C in buffer containing 25 mM HEPES (pH7.5), 50 mM NaCl, 1 mM CaCl<sub>2</sub> and 1% BSA. After incubation, unbound proteins were removed by four consecutive washes with binding buffer lacking BSA. Remaining proteins were eluted by boiling in SDS-PAGE sample buffer and were loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes and the blots probed using an antibody specific for the Fc portion of the receptor fusions. Signals were quantified by scanning densitometry.

### Example 9 - Cell Death Assays

Jurkat cells were resuspended in RPMI/0.5% FBS at  $5 \times 10^4$  cells/well in a 96-well culture dish and treated with vehicle alone, 50 ng/ml Fas Ligand (Upstate Biotechnologies, Inc.), 50 ng/ml TNF $\alpha$  (R&D Systems) or 1  $\mu$ g/ml purified TRDL-1. After 15 h of incubation at 37°C, cells were visualized by light microscopy and photographed. YO-PRO-1 dye (Molecular Probes) was then added at a final concentration of 1  $\mu$ M. After an additional 3 h at 37°C, cells were analyzed on a fluorescence plate reader (Cytofluor II, Perceptive Biosystems) at excitation and emission wavelengths of 485 nm and 530 nm respectively.



To determine a concentration curve, log dilutions of TRDL-1 ranging from 1000 to 0.1 ng/ml were added to cells as described above. After 48 h, cell numbers were quantified using a Coulter counter.

#### Example 10 - Placing TRDL -1 in the TNF family of Cytokines

By searching the dbEST database with sequences of cytokines in the TNF family, a new member of this family, termed TRDL-1, was identified. Although the original EST matches found using TNF $\alpha$  as the search query were weak, their alignments within critical functional domains of the TNF family prompted the close examination of these ESTs. This examination included full sequence determination and offered the first line of evidence for TRDL-1 as new TNF-like cytokine. Sequence analysis of a cluster of TRDL-1 clones obtained from the dbEST clone bank revealed convincing primary sequence similarities with TNF $\alpha$  and other ligands in this family. Primary sequence alignment of ML-1 (Figure 2) with TNF and FasL revealed 25%-43% sequence conservation throughout the length of the proteins with TRDL-1 most closely aligning with FasL. Identities between the TRDL-1 and TNF $\alpha$  and FasL were primarily concentrated to the C-terminal regions of the proteins. These regions facilitate ligand trimerization and interaction with target receptors. Whiteside, T. L. & Rabinowich, H. (1998), *Cancer Immunother.* 46: 175-184; Eck, M. J. et al. (1992), *J Biol Chem.* 267: 2119-2122. Conservation in this region, therefore, defines the TNF ligand family and supports inclusion of TRDL-1 in this family. The level of similarity between TRDL-1 and TNF $\alpha$  typifies comparisons within the TNF ligand family. TRDL-1 shares only 15% identity and 34% sequence conservation with TNF $\alpha$ . Similarly, FasL shares 12% identity and 30% sequence conservation with TNF $\alpha$ .

The general structural organization of TRDL-1 also parallels that of the TNF family. Secondary structure predictions indicated that TRDL-1 is a type II membrane associated protein with a short cytoplasmic tail, a single transmembrane spanning region and an extensive extracellular domain. With the exception of lymphotoxin  $\alpha$ , which lacks an N-terminal membrane spanning helix, each of the TNF family members displays these general structural motifs. Bazzoni, F. & Beutler, B., (1996) *N Engl J Med.* 334: 1717-1725. The expression of recombinant TRDL-1 is confined to the membrane of Cos-1 cells, which supports TRDL-1 as a membrane associated protein,. This suggests that native TRDL-1 exists as a membrane associated molecule like similar cytokines.

TRDL-1 expression was fairly wide spread in normal tissues and is similar to that previously reported for TNF, TRAIL and TWEAK. The highest levels of expression were in peripheral blood leukocytes that also displayed a message size that was unique as compared to other normal tissues. This unique message size could arise from alternate  
5 splicing of TRDL-1 mRNA. The possibility of alternate splicing was confirmed by the identification of TRDL-1 $\beta$  and TRDL-1 $\gamma$ . These two cDNAs were obtained by screening a leukocyte cDNA library with TRDL-1 $\alpha$ . TRDL-1 $\beta$  and TRDL-1 $\gamma$  each contained specific deletions as compared to TRDL-1 $\alpha$  that would result in translation of TRDL-1 $\alpha$ , TRDL-1 $\beta$  and TRDL-1 $\gamma$  messages into three distinct proteins. The size differences in mRNAs seen by  
10 northern analysis correspond closely with size differences between TRDL-1 $\alpha$ , TRDL-1 $\beta$ , and TRDL-1 $\gamma$  cDNAs. Assessment of the functional consequences of alternative splicing warrants further investigation.

Differential expression of TRDL-1 in tumor tissues suggests a potential role for TRDL-1 in tumor development and maintenance. A survey of 48 different tumors compared  
15 to adjacent normal tissues showed increased expression of TRDL-1 in a number of tumors, particularly those derived from the gastrointestinal tract. Tumors from rectum, duodenum, colon, stomach and esophagus showed higher levels of TRDL-1 expression than that seen in adjacent normal tissues. This increased expression in tumors is similar to that seen for expression of FasL. Elevated levels of FasL have been observed in a variety of tumors  
20 including those from the gastrointestinal tract. Shiraki, K. et al. (1997), *Proc Natl Acad Sci USA*. 94: 6420-6425; Niehans, G. A. et al. (1997), *Cancer Res*. 57: 1007-1012. The role of FasL in these tumors is unclear but it may provide a tumor defense against the host immune system or stimulate a chronic inflammatory condition Walker, P. R. et al. (1997), *J. Immunol*.  
25 158: 4521-4524; Whiteside, T. L. & Rabinowich, H. (1998), *Cancer Immunother*. 46: 175-184; Bennett, M. W. et al. (1999), *Gut*. 44: 156-162; Van Antwerp, D. J. et al. (1996), *Science*. 274: 787-789; Wang, C. Y. et al. (1996), *Science*. 274: 784-787. The role for TRDL-1 in tumor development is uncertain but may parallel those proposed for FasL.

Two lines of evidence support functional similarities between TRDL-1 and the TNF family. First, purified, full-length TRDL-1 induced death in Jurkat cells with hallmark  
30 features of apoptosis. Killing of Jurkat cells by TRDL-1 was rapid, saturable and occurred at concentrations typically seen with FasL and TNF $\alpha$ . The rapid rate of death in response to TRDL-1 (within 12 hours) is similar to death induced by FasL and suggests a potential

interaction of TRDL-1 with Fas on Jurkat cells. Secondly, TRDL-1 bound, *in vitro*, to several receptors of the TNF family. TRDL-1 coated beads precipitated purified Fas/fc, TNFR1/fc, HVEM/fc, TR1/fc and TR2/fc fusion proteins. Of these, TRDL-1 preferentially bound Fas/fc and HVEM/fc. This suggests TRDL-1 as an alternate ligand for activating Fas.

5 Although TRDL-1 binds to HVEM, HVEM lacks an intracellular death domain characteristic of apoptosis inducing receptors. Montgomery, R. I. et al. (1996), *Cell*. **87**: 427-436. It is unlikely, therefore, that HVEM mediates the death inducing effects of TRDL-1 in Jurkat cells. Future studies will be necessary to define the cognate receptor for TRDL-1 and to determine under what conditions TRDL-1 may activate Fas or HVEM.

10 Hahne et al. reported the sequence of APRIL, a molecule that is identical in sequence to TRDL-1 $\alpha$ . Hahne, M., et al. (1996), *J Exp Med*. **188**: 1185-1190. They concluded that APRIL is a new member of the TNF family based on structural analyses. These authors reported that APRIL induced cell proliferation and that this proliferative signal could promote tumor cell growth. They propose that this activity is mediated through a novel

15 receptor in that APRIL was incapable of binding purified TNF family receptors *in vitro*. Although reconciliation of the differences between the observations of the present invention and those of Hahne et al. will require additional experimentation, differences in strategies for production of bioactive APRIL/TRDL-1 protein may account for the discordant conclusions. APRIL was produced as a soluble protein that lacked 110 amino acids from its N-terminus.

20 Hahne, M., et al. (1996), *J Exp Med*. **188**: 1185-1190. After the initial identification of TRDL-1, a soluble construct that lacked 53 amino acids from its N-terminus expressed and purified. This truncation removed the membrane-spanning region and allowed for more convenient purification. Addition of truncated TRDL-1, however, to a number of cell lines including Jurkat cells failed to elicit apoptotic responses even at concentrations of TRDL-1

25 as high as 1.0  $\mu$ g/ml (data not shown). We, therefore, turned to production of full-length TRDL-1 and witnessed appearance of death inducing activity as reported herein.

The observed lack of activity of truncated TRDL-1 could result from inability of these molecules to form homo-trimers. Trimerization of TNF family members could be central to the ability of the molecules to efficiently bind to and activate target receptors. Zhang et al.

30 have shown that residues near the membrane spanning helix of TNF $\alpha$  are critical to trimerization of these molecules and biological activity. Zhang, X. M. et al. (1992), *J Biol Chem*. **267**: 24069-24075. Further, Schneider et al. demonstrated that the apoptotic activity

of soluble FasL was reduced 1,000 fold as compared to the membrane bound form of FasL. Schneider, P. et al. (1998), *J Exp Med.* **187**: 1205-1213. However, soluble FasL retained its ability to interact with Fas and restoration of its cytotoxic activity was achieved, both *in vitro* and *in vivo*, with the addition of cross-linking antibodies. Schneider, P. et al. (1998), *J Exp Med.* **187**: 1205-1213. This suggests that the truncated form of FasL is not able to form trimers. Since there are both structural and functional similarities between TRDL-1 and FasL, it is possible that deletion of a significant portion of the APRIL/TRDL-1 N-terminus could alter its activity. It is also possible that truncation of APRIL/TRDL-1 could alter receptor binding specificity and, therefore, elicit different responses. It will be critical to the final assignment of APRIL/TRDL-1 function to determine the trimerization state and receptor binding capabilities of the full-length molecule compared to various N-terminal truncations.

### Summary

In summary, a new member of the TNF family was identified through EST database searching using TNF $\alpha$  protein as the search query. This protein has been termed TNF-Related Death Ligand-1 $\gamma$  (TRDL-1 $\gamma$ ). The nucleic acid molecule coding for the TRDL-1 $\gamma$  has been isolated and purified. This nucleic acid molecule can be cloned into vectors such as plasmids. In the vector, the nucleic acid molecule can be operably linked to a heterologous promoter. A method for screening for cells that undergo apoptosis through interaction with TRDL-1 $\gamma$  is also provided. Agents for inhibiting or enhancing TRDL-1 $\gamma$  mediated apoptosis can also be determined.

The invention may be embodied in other specific forms without departing from its essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. All changes that come within the meaning and range of equivalency of the claims are to be embraced within their scope.